Received: 1 August 2018 | Revised: 10 February 2019 | Accepted: 9 April 2019

DOI: 10.1111/adb.12771

ORIGINAL ARTICLE

WILEY Addiction Biology SSA

Lasting reduction of nicotine-seeking behavior by chronic N-acetylcysteine during experimental cue-exposure therapy

Federico Moro[1] | Giuseppe Giannotti[2] | Lucia Caffino[3] | Claudio Marcello Marzo[4] | Angelo Di Clemente[1] | Fabio Fumagalli[3] | Luigi Cervo[1]

[1] Experimental Psychopharmacology, Department of Neuroscience, Mario Negri Institute for Pharmacological Research—IRCCS, Milan, Italy

[2] Department of Anesthesiology, University of Colorado, Denver, Colorado

[3] Department of Pharmacological and Biomolecular Sciences, Università degli Studi di Milano, Milan, Italy

[4] Department of Biotechnology, University of Verona, Verona, Italy

Correspondence
Federico Moro and Luigi Cervo, Experimental Psychopharmacology, Mario Negri Institute for Pharmacological Research—IRCCS, Via Giuseppe La Masa 19, Milan 20156, Italy.
Email: federico.moro@marionegri.it; luigi.cervo@marionegri.it

Funding information
grants from MIUR Progetto Eccellenza.; Mario Negri Institute for Pharmacological Research IRCCS

Abstract

Nicotine-associated cues can trigger reinstatement in humans as well as in animal models of drug addiction. To date, no behavioral intervention or pharmacological treatment has been effective in preventing relapse in the long term. A large body of evidence indicates that N-acetylcysteine (N-AC) blunts the activation of glutamatergic (GLUergic) neurons in the nucleus accumbens (Nacc) associated with reinstatement. We evaluated the effect of an experimental cue exposure therapy (eCET) alone or in combination with N-AC to verify whether restoring GLU homeostasis enhances extinction of nicotine-associated cues. Rats were trained to associate discriminative stimuli with intravenous nicotine or saline self-administration. Reinforced response was followed by cue signals. After rats met the self-administration criteria, the lasting anti-relapse activity of i.p. N-AC or vehicle was assessed in three different experimental conditions after 14 days of treatment: treatment + eCET; treatment + lever-presses extinction (LP-EXT); and treatment + abstinence. N-AC 100 mg/kg, but not 60 mg/kg, induced anti-relapse activity that persisted 50 days after treatment only when paired with either LP-EXT or eCET with the greater activity found in the latter condition. To identify potential mechanisms for behavioral results, separate groups of rats that received either N-AC or vehicle + eCET were killed at different time points for Nacc Western-blot analysis. Seven days after treatment, chronic N-AC restored the expression of proteins crucial for GLU homeostasis, while at 50 days, it increased the expression of type II metabotropic GLU receptors. These results suggest that N-AC treatment in combination with eCET may offer a novel strategy to prevent relapse in nicotine addiction.

KEYWORDS

drug-associated cues, N-acetylcysteine, nicotine-seeking behavior

1 | INTRODUCTION

An important feature of susceptibility to relapse in drug abuse is the increased salience acquired by environmental stimuli strongly associated with the reinforcing properties of the drug. This has been observed also for nicotine whose consumption engages different Pavlovian and instrumental learning systems in the brain, causing neutral environmental cues and contexts to become strongly associated with its reinforcing properties.[1]

CET (cue exposure therapy) is a nonpharmacological therapy that aims to reduce the impact of nicotine-related cues and prevent relapse. CET relies on extinction training in which drug-associated

cues are repeatedly presented in the absence of the abused drug to promote new learning that should counteract the motivational impact of the cues.[2] Although CET has been proposed to reduce cue-induced nicotine relapse,[3] its efficacy might be limited by the context-dependent nature of extinction therapy[4] and by drug-induced dysfunction of the memory systems that are critical for extinction learning and consolidation.[5] Accordingly, CET has shown limited effectiveness in preventing relapse,[4] and its benefits for nicotine addiction are still debated.[6] To improve CET efficacy, it has been proposed that a more ethological CET targeting drug-associated cues in the same context where the associative over-learning and consolidation took place (ie, becoming "over-learned") could be a better strategy to improve extinction outcome. By using experimental animal models, it is possible to overcome the complexity of the human situation and evaluate whether exposing the animals to the full set of stimuli previously linked to the drug could produce extinction of drug-related memory.

Recent studies have highlighted that changes in glutamate (GLU) homeostasis in the circuitry from the prefrontal cortex (PFC) to the nucleus accumbens (Nacc) contribute to the reinstatement of drug-seeking behavior.[7] In particular, cocaine and nicotine self-administration reduce the expression of the cystine/GLU exchanger (system Xc-), the glial GLU transporter GLT-1 as well as reduce the functionality of group II metabotropic GLU receptors (mGluR2/3),[8] proteins vital for regulating GLU transmission in the Nacc.[9]

Even though acute N-AC administration reduces nicotine-seeking behavior by activating mGluR2/3 and restoring GLU homeostasis,[10] the effect of acute N-AC treatment was short-lasting suggesting that a drug regimen inducing long-lasting repair of nicotine-induced GLU-mediated neuroplasticity might have greater therapeutic value. It has been shown that repeated N-AC consistently restored nonsynaptic GLU tone, normalizing the alterations in the cortico-accumbal synaptic transmission and the activation of glial cells produced by chronic cocaine self-administration.[11] Moreover, repeated N-AC markedly reduced relapse especially when given during the extinction of the instrumental response.[12] For this reason, we hypothesized that restoring nicotine-induced dysfunction in the GLUergic system by repeated N-AC administration could help to increase extinction of the over-learned relationship between nicotine, conditioned-cues, and instrumental response.

In preclinical settings, extinction of drug-related memories has been mostly studied using the extinction/reinstatement model where extinction specifically refers to the extinguishing of the instrumental response used to self-administer the drug.[13] To date, few preclinical studies have evaluated whether increasing the specificity of drug conditioned cues have an impact on cue extinction.[14] With the aim of targeting nicotine-associated cues, we evaluated whether a model of experimental CET (eCET), carried out in a more ethological fashion, alone or in combination with chronic N-AC, could be an effective strategy to extinguish nicotine related memories and whether its effect could be long lasting. Finally, we studied whether key components of GLU transmission in the Nacc were altered by the combination of N-AC + eCET.

2 | MATERIALS AND METHODS

2.1 | Animals

Naïve male Wistar rats (Harlan Laboratories, San Pietro al Natisone, Udine, Italy) weighing 250 to 275 g were used for all experiments. They were housed individually at constant room temperature (21 ± 1°C) and relative humidity (60%) under an inverted light/dark schedule (light on 7:30 PM-7:30 AM) with food and water ad libitum. All experimental work was done during the dark phase. After arriving at the facility, rats were allowed to adapt to the vivarium conditions for at least 2 weeks prior to the start of experiments and were handled daily during this period. After this, they received a maintenance diet of 20 to 25 g/rat of chow/day (Global Diet 2018S, Harlan Laboratories) for the duration of the experiments.

2.2 | Animal care

Procedures involving animals were conducted at the Istituto di Ricerche Farmacologiche "Mario Negri"—IRCCS that adheres to the principles set out in the following laws, regulations, and policies governing the care and use of laboratory animals: Italian Governing Law (D.lgs 26/2014; Authorization n.19/2008-A issued March 6, 2008 by Ministry of Health); Mario Negri Institutional Regulations and Policies providing internal authorization for persons conducting animal experiments (Quality Management System Certificate—UNI EN ISO 9001:2008—Reg. No. 6121); the NIH Guide for the Care and Use of Laboratory Animals (2011 edition) and EU directives and guidelines (EEC Council Directive 2010/63/UE). The Statement of Compliance (Assurance) with the Public Health Service (PHS) Policy on Human Care and Use of Laboratory Animals has been recently reviewed (9/9/2014) and will expire on September 30, 2019 (Animal Welfare Assurance #A5023-01).

2.3 | Drugs

Nicotine was dissolved in saline as previously reported.[10] N-AC was prepared and administered daily i.p. for 14 days as previously described.[10] See the Supporting Information for further details.

2.4 | Apparatus and nicotine self-administration training

Rats were trained in 16 identical operant chambers (ENV-007, MED Associates Inc, St Albans, VT, USA) as previously described.[10] Rats were surgically prepared with jugular catheters and given a week of recovery before self-administration training (see Supporting Information).

One week after surgery, independent groups of rats were food-deprived overnight and trained to associate a white noise (20 dB above background) that lasted throughout the session as a discriminative stimulus (S^{D+}) for the availability of nicotine (0.03 mg/kg/65 μL/2 s/infusion). Rats were not trained for food at the beginning

of the experiment, but they were required to immediately press the active lever under continuous reinforcement (fixed ratio 1 [FR1]) for nicotine self-administration. Sessions started with extension of active and inactive levers, and reinforced response was followed by a light cue (20 s) on the active lever to signal a 20-second time out (CS^+). After 10 days of self-administration training (see Supporting Information for further details), an FR2 was reached and rats were placed on a "discrimination learning" regimen comprising a second daily session without a reward. These sessions started with extension of the active and inactive levers together with illumination of the house light, which remained on throughout the session and served as discriminative stimulus (S^{D-}) for no reward (65 µL/2 s/infusion of sterile saline). Reinforced response was followed by a 20-second intermittent tone (7 KHz, 70 dB) to signal a 20-second time out (CS^-).

The "discrimination learning" phase comprised two daily 1-hour sessions, separated by 1-hour rest in the home cage. Rats were exposed to nicotine and saline sessions in a random sequence. Responses on the inactive lever were recorded but had no programmed consequences. This training was conducted daily for 5 days/week until individual reinforced responding was stable (±15% over three consecutive sessions).

2.5 | First nicotine-seeking test

Twenty-four hours after the self-administration criteria was met, individual animals were tested a first time with either nicotine-associated cues (S^{D+}/CS^+) (half of the rats in each experiment) or

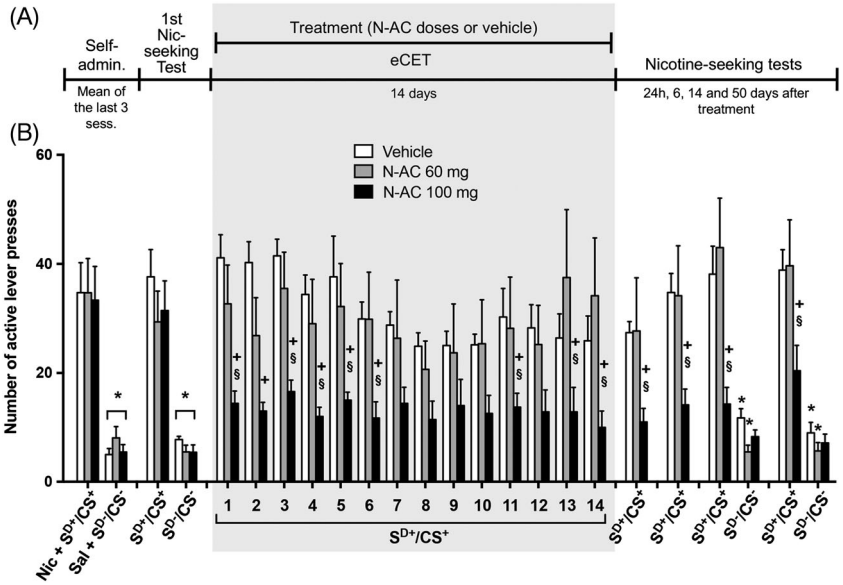


FIGURE 1 Effects of chronic treatment with 60 mg/kg (n = 6), 100 mg/kg (n = 7) N-acetylcysteine (N-AC) or vehicle (n = 8) during eCET on repeated reintroduction of stimuli predictive of (S^{D+}) and associated with nicotine availability (CS^+). (A) Time course of the experiment. After a first reinstatement test, rats were treated daily during eCET. At the end of the treatment, rats were tested with S^{D+}/CS^+ and S^{D-}/CS^- at different time points. (B) Mean ± SEM number of presses on the active lever in a within/between-subject design. Also shown is the number of lever presses during self-administration training (mean ± SEM of last three sessions) and in response to stimuli predictive of and associated with saline availability (S^{D-}/CS^-). $^*P < .05$ vs respective S^{D+}/CS^+, $+P < .05$ vs vehicle, $^§P < .05$ vs 60 mg/kg N-AC–treated group, Newman-Keuls post hoc comparison

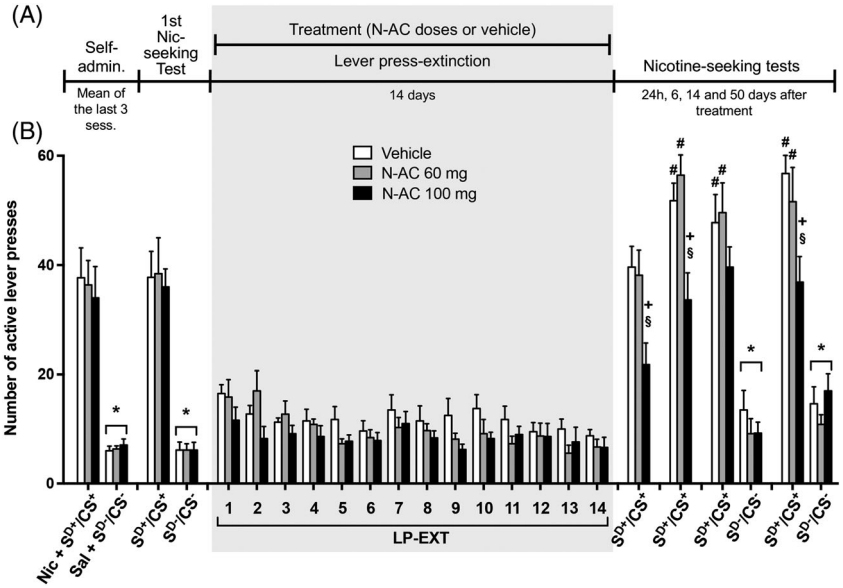


FIGURE 2 Effects of chronic treatment with 60 mg/kg (n = 7), 100 mg/kg (n = 8) N-acetylcysteine (N-AC) or vehicle (n = 8) during LP-EXT on repeated reintroduction of stimuli predictive of (SD^+) and associated with nicotine availability (CS^+). (A) Time course of the experiment. After a first reinstatement test, rats were treated daily during LP-EXT. At the end of the treatment rats were tested with S^{D+}/CS^+ and S^{D+}/CS^+ at different time points. (B) Mean ± SEM number of presses on the active lever in a within/between-subject design. Also shown is the number of lever presses during self-administration training (mean SEM of last three sessions) and in response to stimuli predictive of and associated with saline availability (S^{D-}/CS^-). $^*P < .05$ vs.respective S^{D+}/CS^+, $^#P < .05$ vs respective first S^{D+}/CS^+, $+P < .05$ vs vehicle, $^§P < .05$ vs 60 mg/kg N-AC–treated group, Newman-Keuls post hoc comparison

saline-associated cues (S^{D-}/CS^{-}) (half the rats in each experiment). On the next day, the test order was switched (ie, first day S^{D+}/CS^{+}, second day S^{D-}/CS^{-}, and vice versa). Test sessions lasted 1 hour during which rats were exposed to noncontingent S^{D+} or S^{D-} under conditions identical to the discrimination learning phase, except the reward (nicotine or saline) was unavailable. Two responses on the previously active lever were followed by activation of the pump motor followed by a 20-second CS^{+} or CS^{-} presentation.

2.6 | Effects of the treatments on reintroduction of nicotine-associated cues

Starting 24 hours after the first nicotine-seeking test, chronic treatments with vehicle (veh), 60 or 100 mg/kg N-AC were administered under different experimental conditions:

- eCET (*Instrumental and cue extinction)*: Rats were exposed to the self-administration cage and S^{D+}/CS^{+} under conditions identical to the first nicotine-seeking test. Veh (n = 8), N-AC 60 mg/kg (n = 8), and N-AC 100 mg/kg (n = 8) were given daily for 2 weeks, 2.5 hours before each nicotine-seeking test (Figure 1A).
- Lever press extinction ([LP-EXT], *instrumental extinction)*: Rats were placed in the self-administration cage where no SDs were presented and the instrumental lever response produced neither the reinforcer nor the CSs.[15] Rats were exposed daily for 14 consecutive days to an extinction session of the duration of 1 hour. Responding on either lever had no scheduled consequences. Veh (n = 8), N-AC 60 mg/kg (n = 8), and N-AC 100 mg/kg (n = 8) were given 2.5 hours before each LP-EXT test (Figure 2A).
- Abstinence: Rats were left in their home cage, handled, and injected daily for 14 days with veh (n = 8 rats), N-AC 60 mg/kg (n = 7), and N-AC 100 mg/kg (n = 9) but were not placed in the self-administration cage (Figure 3A).

On days 1, 6, 14, and 50 after treatment, rats were tested with S^{D+}/CS^{+} to assess any effect on nicotine cue-induced seeking behavior. To demonstrate the selectivity of nicotine-associated cues, on test days 14 and 50, half of the rats were tested with S^{D+}/CS^{+} and the other half with S^{D-}/CS^{-}. The day after the test, the order was switched (ie, day 14 S^{D+}/CS^{+}, day 15 S^{D-}/CS^{-}, and vice versa); thus, rats were also tested on days 15 and 51. For the sake of simplicity, we pooled the data from the same cues and indicated them as days 14 and 50 after the end of the treatment (ie, not 14/15 days and 50/51 days).

To assess protein expression in the Nacc, independent groups of rats treated with N-AC during eCET were killed at 7 and 51 days after treatment. Fourteen rats (veh n = 7; N-AC n = 7) were killed 24 hours after the nicotine-seeking tests at 6 days. On the same day, naïve rats that received 14 days of N-AC 100 mg/kg (n = 6) or veh (n = 6) were also killed. Eighteen rats (veh n = 9; N-AC n = 9) were killed 24 hours after the nicotine-seeking tests at 50 days from the end of the treatment. The same day naïve rats that received N-AC 100 mg/kg (n = 6) or veh (n = 6) were also killed.

2.7 | Brain micro-dissection

Rats were killed by decapitation 7 or 51 days after the end of chronic N-AC treatment. Whole brains were frozen on dry ice and stored at −80°C for later micro-dissection. Micro-dissection was done as previously described.[16] Briefly, coronal sections (220 µm thickness) were obtained in a cryostat at −15°C, mounted on glass slides and rapidly cooled with dry ice. Nacc core and shell were microdissected from bregma +2.76 to bregma +0.84 mm according to Paxinos and Watson rat brain atlas[17] a sharp cutting tip of 1 mm diameter (Harris Uni-Core, Ted Pella Inc), snap frozen, and stored at −80°C.

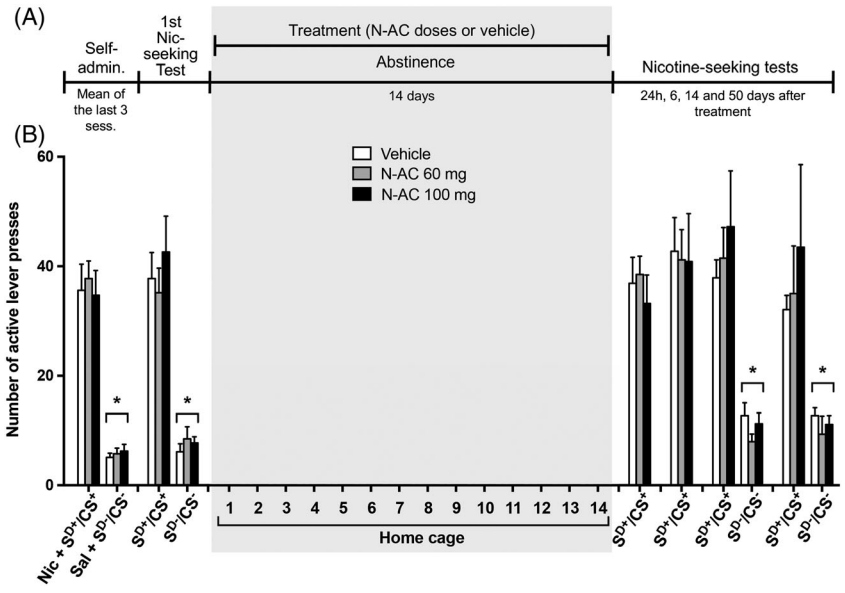


FIGURE 3 Effects of chronic treatment with 60 mg/kg (n = 6), 100 mg/kg (n = 8) N-acetylcysteine (N-AC) or vehicle (n = 8) during abstinence on repeated reintroduction of stimuli predictive of (S^{D+}) and associated with nicotine availability (CS^{+}). (A) Time course the experiment. After a first reinstatement test, rats were treated daily during abstinence. At the end of the treatment, rats were tested with S^{D+}/CS^{+} and S^{D-}/CS^{-} at different time points. (B) Mean ± SEM number of presses on the active lever in a within/between-subject design. Also shown is the number of lever presses during self-administration training (mean SEM of last three sessions) and in response to stimuli predictive of and associated with saline availability (S^{D-}/CS^{-}). $^{*}P < .05$ vs respective S^{D+}/CS^{+} Newman-Keuls post hoc comparison

2.8 | Protein extraction and Western-blot analysis

Protein extraction and Western-blot analyses were performed as previously described.[18] See Figures S1, S2, S3, and S4 for more details and immunoblot data.

2.9 | Statistical analysis

Sample size for each experiment was determined by a combination of power analysis and our previous work in similar models. Animals were randomized to experimental procedures and treatments. Experimenters were blinded to treatment allocation. All data are expressed as mean ± standard error of the mean (SEM) of active and inactive lever presses during the training period, extinction, and nicotine-seeking phases. Only data from rats that completed all experimental phases were included in the statistical analysis. Six rats were excluded: two because of lack of catheter patency, two because the self-administration training criterion was not reached, and two rats were excluded from all the analyses because they were identified as outliers using Grubb's test.[19,20]

In the training period, the number of lever presses during the last three sessions of nicotine self-administration/no-reward was analyzed separately by one-way analysis of variance (ANOVA) with repeated measures. Since there were no differences between sessions, the last 3 days for each condition were pooled for further statistical analysis.

A mixed-factorial ANOVA with treatment as between-subject factor and sessions as within-subject factor was done to analyze the experiments. When appropriate, post hoc comparisons were made with the Newman-Keuls test. Protein levels were analyzed by two-way ANOVA with treatment and self-administration history as main factors. When appropriate, post hoc comparisons were done with Tukey's multiple comparisons test. Assumptions of normality were checked using the D'Agostino-Pearson normality test. Standard software packages were utilized throughout (GraphPad Prism 7, La Jolla, CA, USA).

TABLE 1 Number of self-administration sessions required to meet the criteria (stable reinforced responding ±15% over three consecutive sessions), the mean of nicotine infusions, active and inactive lever presses in the last 3 days of self-administration sessions are reported; numbers of active and inactive lever presses during the first nicotine-seeking test are reported for all the experimental groups

	Self-Administration Sessions (Mean of the Last 3 Days)						First Nicotine-seeking Test			
	Sessions to criteria	Number of Nic. Infusions	Act. Lever Presses		Inact. Lever Presses		Act. Lever Presses		Inact. Lever Presses	
Experiments			Nic	Sal	Nic	Sal	SD^+/CS^+	SD^-/CS^-	SD^+/CS^+	SD^-/CS^-
N-AC + experimental cue exposure therapy (eCET)										
Vehicle	20.3 ± 0.2	13.9 ± 2.5	34.7 ± 5.5*	5.0 ± 1.1	3.3 ± 1.9	5.5 ± 1.7	37.6 ± 5.0*	7.8 ± 0.6	6.4 ± 1.5	8.1 ± 3.0
N-AC 60 mg/kg	20.5 ± 0.2	13.2 ± 2.5	34.7 ± 6.3*	8.1 ± 2.1	1.6 ± 0.4	3.8 ± 1.3	29.3 ± 5.7*	5.5 ± 1.2	3.8 ± 0.9	5.2 ± 3.0
N-AC 100 mg/kg	20.2 ± 0.2	13.5 ± 2.2	33.3 ± 6.2*	5.9 ± 1.3	1.2 ± 0.3	2.5 ± 0.6	31.4 ± 5.5*	5.4 ± 1.4	3.4 ± 1.3	2.4 ± 1.2
N-AC + lever press-extinction (LP-EXT)										
Vehicle	21.5 ± 0.2	16.4 ± 2.1	56.8 ± 9.4*	18.0 ± 4.2	4.4 ± 0.8	3.9 ± 1.1	37.8 ± 4.8*	6.1 ± 1.5	3.3 ± 0.6	4.9 ± 1.5
N-AC 60 mg/kg	21.3 ± 0.2	15.5 ± 1.8	54.4 ± 11.9*	14.5 ± 3.7	5.3 ± 1.9	6.2 ± 1.9	38.4 ± 6.6*	6.1 ± 1.2	6.0 ± 1.7	4.1 ± 1.0
N-AC 100 mg/kg	21.1 ± 0.4	13.8 ± 2.2	58.0 ± 13.3*	16.9 ± 4.2	5.7 ± 1.5	5.8 ± 2.0	36.8 ± 3.3*	6.1 ± 1.2	5.9 ± 1.5	4.9 ± 2.8
N-AC + abstinence										
Vehicle	19.4 ± 0.3	13.8 ± 1.8	35.6 ± 4.5*	6.1 ± 0.6	5.5 ± 1.2	3.6 ± 1.6	33.3 ± 5.5*	5.9 ± 1.3	5.5 ± 1.2	3.4 ± 1.7
N-AC 60 mg/kg	19.7 ± 0.2	15.2 ± 1.5	37.8 ± 3.2*	5.8 ± 1.0	11.1 ± 2.0	5.5 ± 2.9	35.2 ± 4.5*	8.5 ± 2.2	6.6 ± 3.1	3.8 ± 1.8
N-AC 100 mg/kg	19.4 ± 0.3	13.7 ± 2.2	35.6 ± 4.1*	6.3 ± 1.1	6.5 ± 3.1	5.1 ± 1.1	43.0 ± 5.8*	7.7 ± 1.0	11.1 ± 2.0	4.8 ± 1.1
Protein expression										
N-AC + CET (7 d)										
Vehicle	20.3 ± 0.2	14.6 ± 1.4	37.8 ± 4.4*	5.1 ± 0.8	3.3 ± 1.5	4.7 ± 0.8	42.4 ± 10.2*	6.2 ± 1.2	8.4 ± 3.2	4.6 ± 1.0
N-AC 100 mg/kg	20.6 ± 0.2	13.0 ± 2.2	32.4 ± 5.2*	5.3 ± 1.3	2.2 ± 0.5	2.3 ± 0.5	32.9 ± 5.0*	7.0 ± 1.7	3.0 ± 0.8	3.0 ± 0.9
N-AC + CET (51 d)										
Vehicle	20.3 ± 0.2	13.7 ± 2.1	35.1 ± 5.0*	6.0 ± 0.9	1.1 ± 0.5	1.9 ± 0.6	35.6 ± 5.0*	7.0 ± 1.1	1.6 ± 0.4	2.0 ± 0.7
N-AC 100 mg/kg	20.9 ± 0.3	12.9 ± 1.7	31.2 ± 3.6*	5.1 ± 0.7	2.4 ± 0.5	2.4 ± 0.3	30.9 ± 2.8*	5.0 ± 1.1	4.4 ± 2.0	2.9 ± 1.0

Note. Data are expressed as mean ± SEM.

*$P < .01$ vs respective saline or S^{D-}/CS^-, Newman-Keuls post hoc comparison.

3 | RESULTS

3.1 | Training and first nicotine-seeking tests before the beginning of the N-AC treatment

All rats in the different experiments developed stable nicotine self-administration, and lever presses during the last three saline sessions were less than during nicotine sessions ($P < .05$, Newman-Keuls test) and similar across the different groups ($P > .05$, Newman-Keuls test) (Table 1). During the last three self-administration training sessions, all experimental groups earned similar amounts of nicotine, as demonstrated by the similar number of infusions. During the first nicotine-seeking test, S^{D+}/CS^{+}, but not S^{D-}/CS^{-}, renewed active lever presses ($P < .05$, Newman-Keuls test) to similar extents in the different groups ($P > .05$, Newman-Keuls test). The revived active lever presses were similar to those during the nicotine self-administration sessions and significantly higher than during the saline self-administration ($P < .05$, Newman-Keuls test). Inactive lever responses remained negligible throughout all experimental phases.

3.2 | Effect of eCET alone or with chronic N-AC

Figure 1B shows the responses on the active lever during self-administration (mean of the last three sessions), the first nicotine-seeking test, the 14 eCET sessions after veh or N-AC and nicotine-seeking tests at the end of the treatment. Mixed-factorial ANOVA showed significant effects of session ($F_{23,414} = 19.42$, $P < .01$), treatment ($F_{2,18} = 1.47$, $P < .001$) and interaction treatment × sessions ($F_{46,414} = 2.74$, $P < .01$). Responses during the last three nicotine self-administration sessions were similar between groups ($P > .05$, Newman-Keuls test) and significantly higher than the last three saline self-administration sessions ($P < .05$ vs nicotine self-administration, Newman-Keuls test). During eCET + treatment as well as during each nicotine-seeking test, S^{D+}/CS^{+} always revived at the same level the number of active lever presses in veh and 60 mg/kg, but not 100 mg/kg, N-AC ($P < .05$ vs S^{D-}/CS^{-} at the first nicotine-seeking, Newman-Keuls test). In fact, except on test days 7 to 10 and 12, N-AC 100 mg/kg significantly reduced the number of active lever presses, with no tolerance ($P < .05$ vs S^{D+}/CS^{+} and $P > .05$ vs S^{D-}/CS^{-} at first nicotine-seeking and $P < .05$ vs respective veh, Newman-Keuls test). Also, after the treatment + eCET, the reintroduction of S^{D+}/CS^{+}, but not S^{D-}/CS^{-}, always revived active presses in veh and N-AC 60 mg/kg treated rats ($P < .05$ vs S^{D-}/CS^{-} before and after treatment + eCET, Newman-Keuls test). N-AC 100 mg/kg completely blocked the renewed active lever presses induced by nicotine-associated cues ($P < .05$ vs respective veh, $P > .05$ vs S^{D-}/CS^{-} before and after treatment +eCET, Newman-Keuls test).

3.3 | Chronic N-AC treatment during LP-EXT

Figure 2B shows the responses on the active lever during the self-administration, the first nicotine-seeking test, the 14 LP-EXT sessions of rats pretreated daily with veh or N-AC, and nicotine-seeking tests at the end of treatment. Mixed-factorial ANOVA found a significant effect of sessions ($F_{23,460} = 88.20$, $P < .01$) and interaction treatment × sessions ($F_{46,460} = 2.04$, $P < .05$) with no effect of treatment ($F_{2,20} = 2.34$, $P > .05$). Responses during the last three nicotine self-administration sessions were similar between groups ($P > .05$, Newman-Keuls) and significantly higher than the means of last three saline self-administration sessions ($P < .05$, Newman-Keuls test). During LP-EXT sessions, the numbers of active lever presses between days were similar in all groups of rats and always similar to those during S^{D-}/CS^{-} and lower than in presence of S^{D+}/CS^{+} at the first nicotine-seeking test ($P < .05$, Newman-Keuls test). No effect of N-AC was detectable during LP-EXT sessions. After the treatment + LP-EXT S^{D+}/CS^{+}, but not S^{D-}/CS^{-}, revived active presses in all groups ($P > .05$ vs S^{D+}/CS^{+} and $P < .05$ vs S^{D-}/CS^{-} at first nicotine-seeking test, Newman-Keuls test). N-AC 100 mg/kg reduced the renewed active lever presses induced by nicotine-associated cues at 24 hours, 6 and 50 days (but not 14) ($P < .05$ vs veh, Newman-Keuls test). Nevertheless, responses were higher than those observed after saline-associated cues at 14 and 50 days. In veh and N-AC 60 mg/kg treated rats, the active lever presses were significantly higher 6, 14, and 50 days after the end of treatment than during the first seeking test ($P < .05$, Newman-Keuls test), indicating a potential drug-seeking incubation.[21,22]

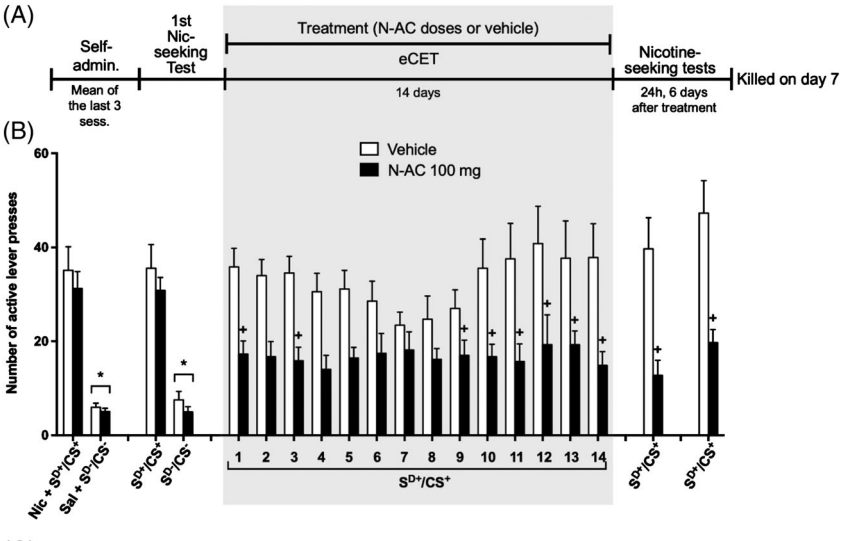
3.4 | Chronic N-AC treatment during abstinence

Figure 3B shows the responses on the active lever during the self-administration and nicotine-seeking tests before and after the end of veh or N-AC treatment. Mixed-factorial ANOVA found a significant effect of session ($F_{9,171} = 40.94$, $P < .001$) with no effect of treatment ($F_{2,19} = 0.08$, $P > .05$) or interaction treatment × sessions ($F_{18,171} = 0.53$, $P > .05$). The number of responses after the reintroduction of S^{D-}/CS^{-} and S^{D+}/CS^{+} differed, as revealed by the main effect of the sessions. N-AC never altered the number of responses during nicotine-seeking tests.

3.5 | Behavioral results and protein analyses of rats killed 7 days after N-AC + eCET

Figure 4B shows the responses on the active lever during self-administration, the first nicotine-seeking test, the 14 eCET sessions of rats pretreated with veh or N-AC 100 mg/kg i.p. and nicotine-seeking tests. Behavioral results are discussed more in details in the Supporting Information, briefly we replicated the results of Figure 1 by showing that during treatment and later nicotine-seeking sessions N-AC 100 mg/kg (but not veh) significantly reduced the number of active lever presses ($P < .05$ vs S^{D+}/CS^{+}, $P > .05$ vs S^{D-}/CS^{-} vs first nicotine-seeking; and $P < .05$ vs respective veh, Newman-Keuls test).

Figure 4C indicates that NAC had an effect on GLT-1 expression in the Nacc shell ($F_{1,22}$self-condition = 2.95, $P > .05$; $F_{1,22}$treatment = 17.13, $P < .05$; $F_{1,22}$interaction = 5.01, $P < .05$). Compared



(C)

Exp. Group	Brain Area	Protein expression					
		GLT1	xCT	mGluR2	GluN1	GluN2B	GluN2A
Naïve/Veh	Nacc-shell	100±5	100±5	100±39	100±10	100±4	100±10
	Nacc-core	100±8	100±6	100±25	100±10	100±2	100±8
Naïve/N-AC	Nacc-shell	111±9	103±14	135±45	109±16	105±8	109±11
	Nacc-core	103±13	112±10	106±28	109±15	90±12	84±14
Nic/Veh	Nacc-shell	**77±2#**	108±8	86±19	136±17	**136±11#**	111±11
	Nacc-core	94±5	118±6	90±35	111±17	78±2	90±7
Nic/N-AC	Nacc-shell	**114±4*****	101±7	69±5	121±10	**103±8***	120±9
	Nacc-core	99±7	113±9	54±6	108±16	72±7	93±12

FIGURE 4 Behavioral results and protein expression in the nucleus accumbens (Nacc) 7 days after the end of chronic treatment with 100 mg/kg N-acetylcysteine (N-AC) or vehicle during eCET. (A) Time course of the experiment. (B) Mean ± SEM number of presses on the active lever in a within between-subject design. Also shown is the number of lever presses during self-administration training (mean ± SEM of last three sessions) and in response to stimuli predictive of and associated with saline availability (S^{D-}/CS^{-}). (C) Western blot analysis expressed as mean ± SEM of rats killed 24 hours after the reinstatement test on day 6 after treatment, Naïve/Veh (n = 6), Naïve/N-AC (n = 6), Nic/Veh (n = 7), Nic/N-AC (n = 7). Behavioral data were analyzed by mixed-factorial ANOVA followed by Newman-Keuls test. Western blot data were analyzed by two-way ANOVA followed by Tukey's test. (B) $^{*}P < .05$ vs respective S^{D+}/CS^{+}, $+P < .05$ vs Veh. (C) $^{*}P < .05$ and $^{***}P < .001$ vs Nic-Veh; $^{\#}P < .05$ vs Naïve-Veh

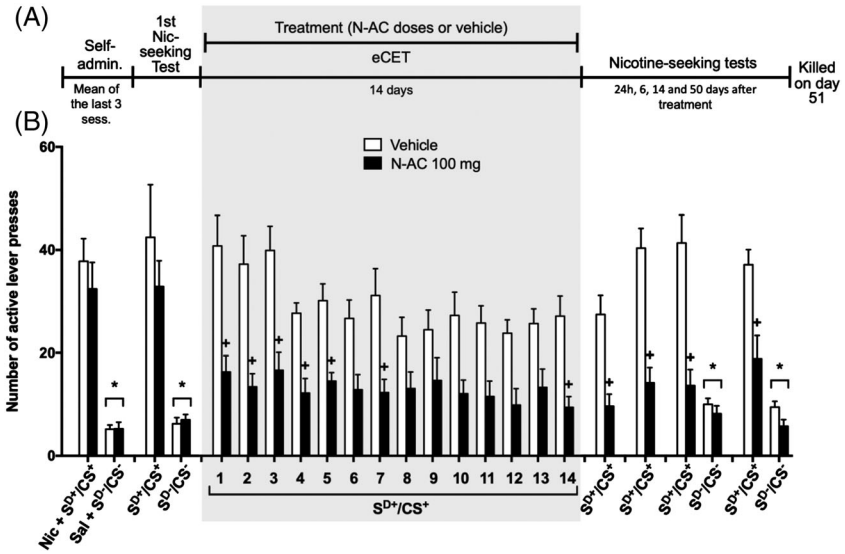
with naïve/veh, the expression of GLT-1 in nicotine self-administered rats treated with veh + eCET was lower ($P < .05$ vs naïve/veh, Tukey's test) and N-AC (nic/N-AC group) restored protein expression to that of naïve rats ($P > .05$ vs naïve/veh, Tukey's test). Moreover, the expression of GluN2B was affected by N-AC in the Nacc shell ($F_{1,22}$self-condition = 2.76, $P > .05$; $F_{1,22}$treatment = 4.11, $P > .05$; $F_{1,22}$interaction = 5.19, $P < .05$). Compared with naïve rats, the expression of GluN2B in nicotine self-administered rats treated with veh + eCET increased ($P < .05$ vs naïve/veh group, Tukey's test) and N-AC (nic/N-AC group) restored protein expression back to the level of naïve rats ($P > .05$ vs naïve/veh, Tukey's test) (Figure 4C). In all experimental condition, the expression of xCT, mGluR2, GluN1, and GluN2A were found similar to those in naïve rats. N-AC did not significantly alter protein expression in naïve animals (Figure 4C).

3.6 | Behavioral results and protein analyses in rats killed 51 days after N-AC + eCET

Figure 5 shows the responses on the active lever during self-administration, first nicotine-seeking test, 14 eCET sessions of rats pretreated with veh or N-AC 100 mg/kg i.p. and nicotine-seeking tests. We replicated the behavioral results of Figures 1 and 4 by showing that N-AC 100 mg/kg (but not veh) reduced active lever presses during treatment and up to 50 days after the end of the treatment (see Supporting Information for more details).

Figure 5C illustrates the protein expression of some determinants of GLU transmission in rats killed 51 days after N-AC + eCET. Nicotine self-administration affected the expression of xCT in the Nacc core ($F_{1,26}$self-condition = 0.004, $P > .05$; $F_{1,26}$treatment = 5.78, $P < .05$; $F_{1,26}$interaction = 7.971, $P < .05$). xCT expression was reduced in nicotine self-administered rats ($P < .05$, Tukey's test) while N-AC restored its expression to the level of naïve rats ($P > .05$, Tukey's test).

N-AC affected the expression of GLT-1 ($F_{1,26}$self-condition = 2.53, $P > .05$; $F_{1,26}$treatment = 1.07, $P > .05$; $F_{1,26}$interaction = 6.07, $P < .05$) and mGluR2 ($F_{1,26}$self-condition = 2.90, $P > .05$; $F_{1,26}$treatment = 3.39, $P > .05$; $F_{1,26}$interaction = 4.3, $P < .05$) in the Nacc shell. Compared with the nic/veh, the expression of GLT-1 and mGluR2 in nicotine self-administered rats treated with N-AC + eCET was higher ($P < .05$, Tukey's test). Moreover, the expression of mGluR2 was higher in the Nacc core ($F_{1,26}$self-condition = 0.37, $P < .05$; $F_{1,26}$treatment = 7.25, $P < .05$; $F_{1,26}$interaction = 4.28, $P < .05$) of Nic/N-AC groups ($P < .05$ vs nic/veh, Tukey's test). In all experimental conditions, the expression of GluN2B, GluN1, and GluN2A was found similar to those in naïve rats. N-AC had no effects on protein expression in naïve animals ($P > .05$, Tukey's test) (Figure 5C).



(C)

Exp. Group	Brain Area	Protein expression					
		GLT-1	xCT	mGluR2	GluN1	GluN2B	GluN2A
Naïve/Veh	Nacc-shell	100±8	100±8	100±5	100±12	100±6	100±9
	Nacc-core	100±7	100±4	100±5	100±5	100±10	100±8
Naïve/N-AC	Nacc-shell	95±7	97±7	93±9	96±13	105±18	87±7
	Nacc-core	105±9	102±9	110±9	106±11	96±13	126±20
Nic/Veh	Nacc-shell	93±3	112±7	96±11	90±8	97±9	86±7
	Nacc-core	105±7	**83±3#**	76±6	108±8	101±9	93±6
Nic/N-AC	Nacc-shell	**115±5#**	109±8	**165±28***	84±11	112±5	111±7
	Nacc-core	105±6	**112± 7*** **	**154±25****	103±13	107±8	87±6

FIGURE 5 Behavioral results and protein expression in the nucleus accumbens (Nacc) 50 days after the end of chronic treatment with 100 mg/kg N-acetylcysteine (N-AC) or vehicle during eCET. (A) Time course of the experiment. (B) Mean ± SEM number of presses on the active lever in a within between-subject design. Also shown is the number of lever presses during self-administration training (mean ± SEM of last three sessions) and in response to stimuli predictive of and associated with saline availability (S^{D-}/CS^{-}). (C) Western blot analysis expressed as mean ± SEM of rats killed 24 hours after the reinstatement test on day 50 after treatment, Naïve/Veh (n = 6), Naïve/N-AC (n = 6), Nic/Veh (n = 9), Nic/N-AC (n = 8). Behavioral data were analyzed by mixed-factorial ANOVA followed by Newman-Keuls test. Western blot data were analyzed by two-way ANOVA followed by Tukey's test. (B) $^{*}P < .05$ vs respective S^{D+}/CS^{+}, $^{+}P < .05$ vs Veh. (C) $^{*}P < .05$ and $^{**}P < .01$ vs Nic-Veh; $^{\#}P < .05$ vs Naïve-Veh

4 | DISCUSSION

This study examined the effects of 14 days of behavioral treatments either alone or in combination with chronic N-AC on cue-induced nicotine-seeking behavior and molecular determinants of GLUergic homeostasis in the Nacc. The main findings were (a) 14 days of eCET, LP-EXT, or abstinence in the home cage did not alter the seeking behavior induced by reintroduction of nicotine-associated cues; (b) 14 days of N-AC (100 mg/kg) treatment during eCET completely blocked nicotine-seeking behavior by associated cues, an effect that lasted at least 50 days after the treatment; (c) 14 days of N-AC (100 mg/kg) treatment during LP-EXT attenuated cue-induced nicotine-seeking behavior up to 50 days after N-AC treatment; (d) 14 days of N-AC treatment during abstinence in the home cage did not alter cue-induced nicotine-seeking behavior; and (e) 14 days of N-AC (100 mg/kg) treatment during eCET changed the expression of proteins vital for regulating GLU homeostasis in the Nacc.

We found that the reintroduction of stimuli predictive of, and associated with, nicotine infusion induced strong and lasting drug-seeking behavior in abstinent rats, as demonstrated in our previous studies.[10,15] This effect cannot be attributed to nonspecific arousal or spontaneous recovery, because responding on the inactive lever and with S^{D-}/CS^{-} remained negligible.

To evaluate the efficacy of our eCET, we refined the procedure we previously used for evaluating antirelapse treatments[10] by removing the extinction phases before and between the nicotine-seeking test sessions. During eCET, rats were exposed to the full set of stimuli (instrumental response and S^{D+}/CS^{+}) associated with nicotine self-administration in a condition identical to that of nicotine-seeking test sessions with the only difference that the treatment was delivered 2.5 hours before the beginning of the sessions. The double self-administration training allowed us to verify at different time points (24 h after nicotine self-administration, 14 days and 50 days after treatment) whether nicotine-related cues (S^{D+}/CS^{+}) induced seeking behavior by comparing the number of active lever presses during the nicotine-seeking test with those produced by the re-introduction of saline-related cues (S^{D-}/CS^{-}). Indeed, in all the nicotine-seeking test sessions, the number of active lever presses were always higher than those elicited by saline-associated cues. The evidence that the limited period of nicotine self-administration (20-22 1-h daily sessions) induced such strong and lasting associations with cues predictive of, and associated with, nicotine availability might be unexpected. However, as with other drugs of abuse,[23-25] drug-associated cues can induce strong, persistent drug-seeking behavior. This is probably because during self-administration sessions, rats were not only exposed noncontingently to the nicotine S^{D+}, signaling drug

availability, but infusions were also paired with a response cue marking the 20-second time-out period acting as CS^+. Thus, reintroduction of S^{D+} by signaling drug availability may favor the search of the substance, and the contingent CS^+ may subsequently have maintained drug-seeking behavior.[15,25] Moreover, nicotine acts not only as a primary reinforcer but also as a reinforcement enhancer, as demonstrated in both preclinical[26-28] and clinical studies[29]: this may explain the increased salience for nicotine-related stimuli.

A further interesting result of our study was that, even though eCET was carried out in "a more ethological way" (ie, with the same stimuli presented in the same context), it was not sufficient to reduce the salience of nicotine-associated cues during the 14 days of treatment nor during the later nicotine-seeking tests. This was in line with clinical studies reporting that CET for tobacco cessation was less successful in promoting cessation when compared with other drug dependencies.[4] By contrast, when eCET was performed in combination with repeated N-AC 100 mg/kg, nicotine-seeking behavior was blocked throughout the 14 treatment days, and its action lasted for at least 50 days after treatment. Also, when N-AC 100 mg/kg was given in combination with LP-EXT sessions, it reduced nicotine-seeking behavior at 24 hours, 6 and 50 days (but not 14). Nevertheless, N-AC + LP-EXT only attenuated nicotine-seeking behavior since at test days 14 and 50 active lever presses during the presentation of nicotine-associated cues were higher than those observed after saline-associated cues. The reasons for these different activities of N-AC are not known, and future investigation will address this important issue. It could be argued that N-AC produces a generalized attenuation of the response; however, this seems unlikely since 100 mg/kg N-AC given during abstinence failed to modify nicotine-seeking tests. Thus, N-AC seems to be more active when given in combination with the full set of stimuli (instrumental response and S^{D+}/CS^+) associated with nicotine self-administration than given in combination with part of them (instrumental response). This result seems to be in line with a study from Reichel et al.[12] where they found that repeated N-AC exerts more profound effect in preventing cocaine cue-induced seeking behavior when given during LP-EXT than during abstinence. A direct comparison between their findings and ours is limited by several experimental differences (ie, the type of drug of abused and the training paradigm). Nevertheless, it is also important to note that the contingency by which N-AC treatment is delivered seems to affect N-AC anti-relapse activity across different type of drug of abuse.

The precise mechanisms by which N-AC + eCET blocked cue-elicited nicotine-seeking is not known yet. One possible explanation may lie in the nicotine-induced alteration of GLUergic homeostasis in brain regions known to affect cue-induced seeking behavior, thus impairing the ability to extinguish nicotine-related cues. Thus, it is possible that repeated N-AC treatment restored the "top-down" GLUergic control over seeking behaviors, promoting the extinction of nicotine-associated stimuli only when N-AC treatment is paired with the cues. This hypothesis is further supported by the lack of effect of chronic N-AC treatment when administered during abstinence. Supporting this interpretation, repeated N-AC treatment during extinction sessions after cocaine self-administration has previously demonstrated lasting anti-relapse activity.[11,12,30] A second interpretation could be that nicotine has enhanced responding for the unconditioned reinforcing stimuli (S^{D+} and/or CS^+) and that N-AC-induced decrease of S^{D+}/CS^+ responding during and after eCET would not necessarily result from a facilitated extinction but rather by acutely reducing the reinforcing value of S^{D+}/CS^+. However, this seems to be unlikely since N-AC was found to still be effective 14 and 50 days after the end of the treatment, and previous work has shown that chronic N-AC also reduced heroin[31] and cocaine[12] cue-induced seeking behavior.

To the best of our knowledge, this study describes the first attempt to dissect the long-term antirelapse effect of repeated N-AC when given during eCET, rather than LP-EXT, on nicotine-seeking behavior. Our results seem to be in agreement with those obtained after cocaine[11] and heroin[31] self-administration.

To investigate the molecular correlates mediating the long-lasting anti-relapse effect of N-AC in combination with eCET treatment, we examined the expression of proteins associated with GLU homeostasis in the Nacc, since evidence exists of overlapping effects on GLU transmission in this brain region after extinction training[32] and after repeated N-AC.[11,30] First, even in this cohort of rats, we have replicated behavioral findings, showing that N-AC + eCET decreased cue-induced nicotine-seeking behavior during the treatment while maintaining anticraving activity. Next, we sought to quantify changes in proteins crucial for GLU homeostasis as these are consistently altered across different drugs of abuse and might account for the lasting behavioral responses produced by drug-related cues.[33] Previous studies have reported a decrease in the expression of xCT[9] and GLT-1,[9,34] and high expression of the GluN2B subunit of NMDARs[34] in the Nacc after withdrawal from nicotine self-administration. xCT and GLT-1 were also lowered during withdrawal from cocaine[30,35] and ethanol[36] self-administration.

Interestingly, 7 days after eCET, we found lower expression GLT-1 as well as higher expression of GluN2B in the Nacc shell of the nicotine self-administered rats when compared with naïve rats. Repeated treatment with N-AC, with eCET, restored the GLUergic deficits mediated by nicotine self-administration. It is important to note that protein expression showed no significant changes in the Nacc core of the same animals. The fact that the expression of these proteins was different in the two subregions of the Nacc is not surprising since Nacc is a very complex area that mediates the reinforcing effect of drugs of abuse and integrates cognitive and affective information processed by cortical regions.[37] The Nacc shell receives GLUergic afferents from the infralimbic cortex and activation of this pathway promotes extinction[38] alongside attenuating cue-induced reinstatement of cocaine-seeking behavior.[39] The Nacc core receives GLUergic afferents from the prelimbic cortex, and this pathway is activated during cue-induced drug-seeking behavior.[40] Although the Nacc core is the region that has been implicated mostly in drug-seeking behavior,[7] recently, the Nacc shell has emerged as the subregion involved in reward devaluation and extinction of drug-related cues.[41] Thus, restored GLU homeostasis in the Nacc shell may account for the extinguishing/attenuation of the tendency of nicotine-associated cues

to induce seeking behavior in rats treated with N-AC + eCET. In the present experiment, rats were not only treated with N-AC but were also exposed daily to eCET: accordingly, the results should be viewed as a combination of a pharmacological (N-AC) and behavioral (eCET) treatment.

Interestingly, 51 days after the end of the N-AC + eCET treatment (ie, more than 2 months after the last nicotine self-administration session), the protein profile in the Nacc was drastically different. The only considerable effect induce by nicotine self-administration was a reduced expression of xCT in Nacc core, while N-AC + eCET bring back xCT expression to naïve level. In contrast, N-AC treated rats displayed a steep increase in mGluR2 and GLT-1 expression, perhaps as an attempt to tone down the increased GLU release.

The differences in protein expression at different time points suggest that behavioral and molecular results are not directly correlated. To the best of our knowledge, protein levels in the Nacc at chronic time points after nicotine self-administration and N-AC + eCET have not yet been investigated. It is possible that 7 days after the end of the treatment, nicotine-induced changes in protein expression were still present while the treatment was reversing them. Conversely, 51 days later, the overexpression of mGluR2 might counteract the increased GLU release in the Nacc caused by the activation of PFC afferents during cue-induced nicotine-seeking tests. Moreover, the increased expression of GLT-1 in the Nacc shell and the increased expression of xCT in the Nacc core might further prevent activation of post-synaptic receptors, thus blunting GLU transmission.

In conclusion, the present results support the validity of N-AC treatment in nicotine addiction and identify eCET as a major contributor to the mechanisms that improve the outcome of N-AC in preventing relapse. In addition, taking into account that evidence for N-AC treatment for nicotine addiction in humans is still limited[42] and that, so far, human studies have evaluated the efficacy of repeated N-AC treatment during abstinence[43] or while the subjects were still taking nicotine,[9,44-46] our data pave the way for a novel approach of N-AC, in combination with CET, for clinical trials.

ACKNOWLEDGEMENT

This research was supported by Mario Negri Institute for Pharmacological Research IRCCS and grants from MIUR Progetto Eccellenza.

ORCID

Federico Moro https://orcid.org/0000-0002-2682-5583
Claudio Marcello Marzo https://orcid.org/0000-0002-2249-4692
Fabio Fumagalli https://orcid.org/0000-0002-8814-7706

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

How to cite this article: Moro F, Giannotti G, Caffino L, et al. Lasting reduction of nicotine-seeking behavior by chronic N-acetylcysteine during experimental cue-exposure therapy. *Addiction Biology*. 2020;25:e12771. https://doi.org/10.1111/adb.12771